Immobilized laccase on Polyimide Aerogels for removal of carbamazepine

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Abstract

Since it is known that conventional wastewater treatment plants cannot completely remove pharmaceutical compounds, such as carbamazepine, the need for their removal has intensified. The use of biocatalysts, such as enzyme is an environmentally friendly method for carbamazepine biodegradation. Nevertheless, enzyme immobilization is required to facilitate the recovery and reusability and avoid the loss of enzyme. In this work, laccase was immobilized on modified polyimide aerogels by means of covalent bonding. Results showed that the immobilized laccase on polyimide aerogels possesses significantly improved activity under acidic or basic pH range in comparison with the free enzyme. Furthermore, for all the temperature range the activity of the immobilized enzyme was higher compared to the free enzyme form. The storage stability improved by the immobilization on this support material. The reusability tests towards oxidation of 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) showed that the immobilized laccase maintained 22% of the initial activity after 7 cycles. Immobilized laccase on polyimide aerogels for carbamazepine (CBZ) degradation exhibited 76% and 74% removal in spiked water and secondary effluent, respectively. Furthermore, after 7

cycles the CBZ removal efficiency remained higher (50% and 65% for spiked water and secondary effluent, respectively).

Keywords: Polyimide, Aerogels, covalent Immobilization, Laccase.

Introduction

Pharmaceutical compounds in the aquatic environment have received global concerns due to their health risk to humans and impact on the aquatic life [1-3]. Furthermore, it is known that conventional wastewater treatment plants cannot completely remove these compounds [4, 5]. Among them, carbamazepine (CBZ), an anticonvulsant and mood-stabilizing drug used in the epilepsy treatment and bipolar disorder [6], is one of the most frequently detected pharmaceutical compounds in environmental systems [7,8]. Many treatments techniques exist for the removal of CBZ, such as membrane filtration, advanced oxidation processes, and adsorption [9,10]. One of the most efficient methods for the removal of CBZ is oxidation but has several disadvantages, such as incomplete mineralization, requiring continuous supply of O_3 or H_2O_2, requiring O_2 and/or H_2O_2 storage, post-treatment to remove potentially toxic catalysts and the risk of adsorption on the catalyst surface rather than mineralization [11]. As an alternative, the use of biocatalysts, such as enzymes could be a good option [12]. Enzymes are bimolecules that can mediate reactions without severe conditions and with benign by-products formed during the catalysis [12-14]. Laccase enzymes can oxidize a wide range of substrates. Furthermore, laccase can oxidize, polymerize or transform several substrates into less toxic compounds. Thus, laccases provides an environmentally friendly technique and possess lower energy consumption and moderate operational conditions in comparison with the other treatment techniques for the CBZ removal [12-14]. In general, an efficient method for enzyme immobilization is necessary to facilitate the recovery and reusability and avoid the loss of enzyme [15-19]. The most common

methods for laccase immobilization are covalent, adsorption, cross-linking, encapsulation, and entrapment [20]. The covalent attachment is the most commonly used method as it prevents enzyme leaching and improves enzyme stabilization. Moreover, it provides stable and strong attachment and is more stable in the reaction system in comparison with other immobilization techniques [21-23].

The support should protect the enzyme structure of the severe reaction conditions and helps the immobilized enzyme to retain high catalytic activity.[24] Furthermore, the affinity between the functional groups of the enzyme and the support must be higher to create stable enzyme-matrix interactions and effective binding of the enzyme to the support [25].The main characteristics of the support materials to get effective enzyme immobilization are chemical and thermal stability, insolubility under reaction conditions, high affinity to enzymes, biocompatibility, the presence of reactive functional groups, regeneration, and reusability and appropriate availability and cost [25]. Since it is known that inorganic supports have several limitations, such as limited biocompatibility, lower affinity to biomolecules and reduced possibilities to create various geometrical shapes, the use of organic materials as supports has intensified [26] The use of synthetic polymers has advantages because the monomers that build the polymeric chain can be selected according to the requirements of the enzyme and process [25]. These materials have numerous functional groups, such as carbonyl, carboxyl, hydroxyl, epoxy, amine and diol groups, as well as strongly hydrophobic alkyl groups and trialkyl ammine that facilitate enzyme binding and the functionalization of the polymer surface [25]. In this regard, polyimide could be a good option as support material for enzyme immobilization as a consequence of its excellent mechanical, dielectric and thermal properties. [27]. Polyimide aerogels could be a good alternative as support material for enzyme immobilization due to their physicochemical

properties (high porosity and very low density, mainly), thermal stability, the possibility of modify their surface to carry out the covalent attachment, their mechanical and chemical stability and the controlled hydrophobicity [27, 28] Furthermore, the methodology for the production of the polyimides aerogels used in this work is based on an environmental friendly freeze-drying process [29]. Paşahan et al. [30] synthesized naphthalen based polyimide from five commercial dianhydrides to be as a membrane for immobilization of the glucose oxidase. Polyimide membranes presented good behavior as a consequence of the high chemical stability and selectivity.

Çakmakçi et al. [31] prepared polyimide membrane to carry out the covalent immobilization of α-amylase. Results showed that the immobilized enzyme presented more stable character in comparison with the free one.

To the best of our knowledge, this is the first report on covalent immobilization of laccase on modified polyimide aerogels. Free enzyme activity and immobilized enzyme activity were carried out at different pH and temperatures. Moreover, the immobilization efficiency was analyzed. Furthermore, the reusability and storage stability were studied as well as the performance of immobilized laccase for the removal of CBZ.

2. Materials and Methods.

2.1. Materials.

4,4'-Oxydianiline (ODA),3,3'4,4'biphenyltetracarboxylicdianhydride (BPDA) , 1-methyl-2-pyrrolidone (NMP) and tryethylamine (TEA) were purchased from Sigma–Aldrich. Ethylenediamine, methanol, and glutaraldehyde (25%) were also purchased from Sigma-Aldrich as used as received.2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonicacid) (ABTS) and carbamazepine (CBZ) with ≥99% purity were purchased from Sigma-Aldrich (Oakville, ON, Canada). Tween 80 was obtained from Fisher

Scientific (Ottawa, Canada). Carbamazepine(D10), as an internal standard in mass spectroscopy, was purchased from CDN Isotopes (Pointe-Claire, Canada). Apple pomace (VergersPaul Jodoin Inc., Quebec, Canada) was used as a solid substrate for laccase production using *Trametesversicolor* (TV). Secondary effluent [32] samples were collected from Quebec wastewater treatment plant (Beauport, Quebec City, Canada). Ultrapure (double distilled) water was produced in the laboratory usingMilli-Q/Milli-RoMilli pore system (Massachusetts, USA).

2.2. Extraction and production of the enzyme.

Laccase production and extraction were carried out as reported in previous works [32]. For the enzyme preparation, forty grams of apple pomace with 78% (w/w) moisture and pH 4.5 were mixed with Tween 80 (0.5% v/w) in 500 mL Erlenmeyer flasks and autoclaved at 121 ± 1 °C for 20 min. After that, the substrate was inoculated with *Trametesversicolor* (ATCC 20869) and incubated at 30 ± 1 °C for15 days.

For enzyme extraction, one gram of fermented sample was mixed with 20mL of 50mM sodium phosphate buffer (pH6.5). After the mixture homogenization on incubator shaker at 35±1 °C and 150 rpm for 1 h, it was centrifuged at 7000 ×g for 30min. The collected supernatant was analyzed for enzyme activity and dried under vacuum (5 Pa) at −55 °C for 48 h using freeze dryer (FD-1000, Eyela, Japan).

2.3. Preparation of polyimide aerogels.

Water-soluble poly (amic acid) (PAA), the precursor of polyimide was synthesized from a polycondensation reaction (as shown in Figure 1) between aromatic acid dianhydride (BPDA) and aromatic diamine (ODA) procedure in 1-methyl-2-pyrrolidone (NMP) using an equivalent molar ratio of 4,4'-ODA and BPDA at 0-5^{o}C. Then PAA solution was precipitated with deionized water and the precipitate was washed several times with water and dried under vacuum.

A certain amount of tryethylamine (TEA) was first dissolved in deionized water, the solution was continually stirred in an ultrasound bath. Then 3 wt. % dried PAA was added to the TEA-water solution and stirred. Then, PAA/TEA- water solution was introduced into the trays of the laboratory freeze-dryer, frozen at -60°C and, finally, sublimed under vacuum to obtain PAA aerogel. Later, PAA aerogel was placed in a vacuum oven to carry out the thermal imidization to obtain polyimide aerogel. The color was changed from white to bright yellow. Thermal imidization was confirmed by Fourier Transform Infrared Spectrum (FTIR).

2.4. Surface modification of polyimide aerogels.

Polyimide aerogels were modified similar to reported procedures [31, 33-35]. The amination of polyimide aerogels was carried out by immersing the aerogel in a solution of ethylenediamine in methanol (10% w/v) for 30 min. Afterwards, the modified aerogel was washed immediately with methanol to eliminate the residual ethylenediamine. Later, the modified aerogel was dried at 60±1°C for 24 h. for the complete removal of methanol. Figure 2 shows the surface modification of polyimide.

2.5. Immobilization of laccase on polyimide aerogels.

Surface modified polyimide aerogels were immersed in a solution of glutaraldehyde (25%) under vigorous stirring at room temperature for 1 h. Then, aerogels were removed with distillated water to removed unreacted glutaraldehyde. After that, glutaraldeyde activated polyimide aerogels were dried at 60ºC for 10 h. In 50 mL flasks, glutaraldeyde activated polyimide aerogels were suspended in 10 mL of citrate-phosphate buffer (pH 4.0) containing a known amount of laccase. The covalent immobilization process was carried out over 12 h with shaking at 400 rpm at room temperature. Figure 3 shows the enzyme immobilization process on the polyimide

aerogels. The laccase activity in the supernatant and also in immobilized laccase on polyimide aerogels was determined.

On the other hand, immobilization of laccase using adsorption was carried out to compare the obtained results. Polyimide aerogels were suspended in 10 mL of citrate-phosphate buffer (pH 4.0) containing a known amount of laccase. The immobilization was carried out for 12 h. with shaking at 400 rpm at room temperature. The laccase activity in the the supernatant and also in immobilized laccase on polyimide aerogels was determined.

2.6. Enzyme Assay.

In this work, the activity of the free enzyme and immobilized enzyme on polyimide aerogel was determined through monitoring the rate of oxidation of ABTS. One unit of laccase activity was defined as the amount of required enzyme to oxidize one μmol of ABTS per min under the assay conditions.

For free enzyme, the reaction mixture contained 2.450 mL citrate-phosphate buffer (pH 4.0), 500 μL ABTS (1.5 mM) and 50 μL of laccase sample. The oxidation of ABTS at 45ºC was monitored by an increase in absorbance at the wavelength of420 nm ($\varepsilon 420 = 36 mM^{-1} cm^{-1}$) [32].

For immobilized laccase, a known amount of sample (polyimide aerogel after laccase immobilization on its surface) was mixed with 3 mL of citrate phosphate buffer (pH 4.0) and reacted for 10 min with one mL ABTS (1.5 mM, pH 4) at 45ºC and 200 rpm. Then, the absorbance of the supernatant was measured at 420 nm and the final activity of laccase immobilized on polyimide aerogel was expressed as Unit/g polyimide aerogel.

To determine the pH and temperature profiles for the free and immobilized laccase activity, assays were carried out over the pH range of 3.0–10.0 and temperature range of 20–50°C.

2.7. Reusability.

A known amount of sample (polyimide aerogel after laccase immobilization on its surface) was dispersed in 3 mL of citrate-phosphate buffer (pH 4.0) and incubated with 1 mL of ABTS for 10 min at 45ºC. At the end of the reaction, an immobilized enzyme on polyimide aerogel was washed with distilled water and then added the buffer and ABTS solution to start a new cycle. The procedure was repeated for 7 cycles.

2.8. Effect of storage time on enzyme activity.

For storage stability, the free and immobilized laccase samples were stored at room temperature for up to 20 days and residual activities were determined at 5-day intervals.

2.9. CBZ degradation by laccase immobilized on polyimide aerogels.

2.9.1. Repeated use of immobilized laccase on polyimide aerogels.

The behavior of immobilized laccase on polyimide aerogels as materials to remove CBZ from aqueous media was evaluated in batch tests in both Milli-Qwater and secondary effluent (wastewater treatment plant).In a 50-mL flask, a known amount of immobilized laccase on polyimide aerogel was dispersed in 20 mL of CBZ solution (20 ng/mL) and the reaction mixture was stored at 200 rpm and room temperature for 24 h because previous tests indicated that after 24 h, the removal rate was negligible. The supernatant was decanted (10 min and 11, 000 ×g) and CBZ removal efficiency was measured based on its initial and final aqueous phase concentrations. The immobilized laccase on polyimide aerogel was washed with Milli-Q water, decanted and the procedure was repeated.

2.9.2. Removal of CBZ.

Solutions with 30 mL of milli-Q water, a known amount of immobilized laccase on polyimide aerogels and CBZ solution (20 ng/mL) were incubated at 25 ºC at 200 rpm for 24 h. After that, the CBZ concentration was measured. Furthermore, to determine the CBZ amount adsorbed on immobilized laccase on polyimide aerogels, freeze dried samples were mixed with 5mL of methanol, sonicated for 10 min and incubated for 8 h at room temperature at 200 rpm to desorb CBZ. Finally, the mixture was decanted and the concentration of CBZ in methanol was measured [32].

2.10. Characterization.

FT-IR spectra were recorded (Nicole IS50 FT-IR Spectrometer -Thermo Scientific, USA-) in the range of 400–4000 cm^{-1} to analyze the chemical interactions in polyimide aerogels after the surface modification and enzyme immobilization.

Cary 50 UV–visible spectrophotometer (Varian, Australia) was used to determine the oxidation of ABTS by an increase in absorbance at the wavelength of 420 nm ($\varepsilon 420$ = $36 mM^{-1}$ cm^{-1}).

Laser Diode Thermal Desorption (LDTD) (Phytronix technologies, Canada) coupled with an LCQ Duo ion trap tandem mass spectrometer (Thermo Finnigan, USA) was used to CBZ quantification. The daughter ions identified for CBZ in LDTD were 194 and 192 Da. A calibration curve of CBZ concentration was developed with six standard solutions and with R^2 no<0.99. All the experiments were performed 3 times and the average results were reported.

2.11 Statistical analyses

All the experiments were performed in triplicates, and the average of replicates and standard deviation were determined. Analysis of variance (ANOVA) was performed for

the data using Microsoft Excel 2013 and the results which have $P < 0.05$ were considered as significant.

3.Results and Discussion

3.1. Characterization of polymeric support material

The chemical modifications of the polymeric support material was sought to create reactive amino groups via the amination reaction with ethylenediamine. The electrophilic imide group of polyimide reacts with amine nucleophilic agent and consequently, the imide ring is opened, and an amide is formed [31]. Then, for the enzyme bonding, amino groups of the surface of polyimide were activated by glutaraldehyde. The aldehyde group links to the classical Schiff´s base with the amino groups, leaving the distal aldehyde group available for covalent enzyme coupling [31].

The FTIR spectra of polyimide aerogel, aminated polyimide aerogel, aminated polyimide aerogel activated with glutaraldehyde and enzyme immobilized on polyimide aerogel is shown in Figure 4. Polyimide aerogels clearly indicated the presence of imide ring. The characteristic absorption bands of imide ring that appeared at 1775 cm^{-1} attributed to the asymmetrical carbonyl stretching vibration and 1720 cm^{-1} to the symmetrical carbonyl stretching vibrations. Furthermore, the peak around 1370 cm^{-1} is attributed to C-N bond stretching and the peaks 1050 and 723 cm^{-1}corresponded to imide ring deformation. In the spectrum of aminated polyimide aerogels, the peaks at 1690 cm^{-1} (C=O stretching) and 1538 cm^{-1}(N-H bending) appeared as a consequence of the amide formation. For aminated polyimide aerogels activated with glutaraldehyde, the spectrum shows a peak around 1650 cm^{-1} which was attributed to the imine groups due to interaction between amino groups and glutaraldehyde [31]. In the spectrum of laccase immobilized on polyimide aerogel, the amide I and II bands of the laccase were

observed at 1630 cm^{-1} and 1500 cm^{-1}. The amide I at 1630 cm^{-1} was attributed to C=O stretching vibrations and the amide II at 1500 cm^{-1} is primarily attributed to NH bending vibration and CN stretching vibration in laccase. Thus, amination, activation, and immobilization was achieved successfully.

3.2. Laccase immobilization on polyimide aerogels.

Laccase was immobilized onto polyimide aerogels using different immobilization methods. Table 1 shows the laccase activity, the binding efficiency and the effective binding efficiency of immobilized laccase on polyimide aerogels. The binding efficiency represents the theoretical activity (the difference between the activities in the liquid phase before immobilization and after washing step)of the bound laccase to the support divided by the initial laccase activity, resulting in 0.46 and17.2 % for adsorption and covalent, respectively.The effective binding efficiency is defined as the apparent activity divided by the initial laccase activity during immobilization, resulting in 1.43 and 5.2 % for adsorption and covalent, respectively. As expected, covalent immobilization was more effective than adsorption. It is known that covalent immobilization provides strong and stable enzyme attachment. Furthermore, it prevents enzyme leaching and improves enzyme stabilization [25]. As a consequence of the higher effective binding efficiency of covalent immobilization (17.2 % in comparison with 0.46% for adsorption), this attachment method was employed for the rest of the study.

3.3. Effect of pH on enzyme activity.

The effect of pH on the free and immobilized laccase was studied in the range of pH 3.0 to 10.0 and results are shown in Figure 5. According to these results, at pH 3.0, both free and immobilized laccase showed the highest activity. Furthermore, it can be seen that the immobilization of laccase onto polyimide aerogels made laccase much more

resistant to the acidic environment [11] (below pH 5.0). Moreover, at pH from 6.0 to 10.0, the activity of laccase considerably improved with the immobilization. These results showed that the immobilized laccase on polyimide aerogels possesses significantly improved activity under acidic or basic pH range in comparison with the free enzyme as a consequence of the high chemical stability of the polyimide aerogel [27]. Other authors found that the immobilization of *Rhyzopus oryzae* lipase onto silica aerogels increased its stability in a wade pH range [36]. Furthermore, the activity decreasing over the pH range could be due to the interactions between the polyimide aerogel and the amino acid groups at the active sites of the enzyme.

3.4. Effect of temperature on enzyme activity.

The activity of the free and immobilized laccase was analyzed at various temperatures (20–50°C). As it can be seen in Figure 6, for all the temperature range the activity of the immobilized enzyme was higher compared to the free enzyme form. It means that the immobilized method preserved the enzyme activity. This behavior is due to the covalent bond formation via amino groups that might reduce conformational flexibility and therefore the immobilized laccase on polyimide aerogel became more resistant to heat inactivation [11, 21, 36].

3.5. Storage stability.

The storage stability of free and immobilized laccase was evaluated at room temperature for up to 20 days and residual activities were determined at 5-day intervals. The effect of storage stability of the immobilized and free enzyme is an important aspect to ensure a long shelf life. Generally, the enzyme in its free form is not stable during storage and gradually decrease its activity [32]. Results are shown in Figure 7 and indicated, as expected, that the immobilized laccase on polyimide aerogels had better storage stability than free laccase during 20 days of storage. During the first storage period (5 days),

26% activity reduction was observed for immobilized laccase and 58% for free laccase. Furthermore, it can be seen that after 20 days of storage, free laccase showed 96% of activity reduction while immobilized laccase still had 20% of its initial activity. The increase of activity when the enzyme was immobilized on polyimide aerogel was attributed to stabilization of the enzyme on support, structural rigidness and protection of enzyme from unfolding and denaturation as reported in other works [32, 37,38].

3.6. Operational Stability.

The operational stability of the immobilized laccase was investigated for seven consecutive cycles of ABTS oxidation, and the results are shown in Figure 8. The remaining activity of the immobilized laccase on polyimide aerogel decreased with the cycles number increase. At the end of the 5^{th} cycle, the residual activity was 22% of its initial activity and remained almost constant until 7^{th} cycle. The decrease in enzyme activity with the cycles could be due to leaching and/or denaturation of the enzyme during the reactions [32, 39].

To evaluate the operational stability of the immobilized laccase on polyimide aerogels for the removal of pharmaceutical compounds, CBZ from the ultrapure and secondary effluent of wastewater treatment plan was investigated and the results are shown in Figure 9. As shown, the CBZ removal after 7 cycles decreased from 76% to 50% and 74% to 65% for ultrapure and secondary effluent, respectively. The CBZ degradation performance of biocatalyst in secondary effluent was higher than in ultrapure water, probably due to the presence of ions in secondary effluent that induce structural modifications in the active site of the enzyme. These ions present in effluent are in favor of electron transfer in electrochemical reactions [32]. The CBZ removal efficiency decrease was due to the high molar mass of the products formed during the oxidation of CBZ that lead to higher mass transfer resistance [6], and the leaching and denaturation

of laccase [32]. Figure 10 shows the percentage of CBZ removed as a consequence of the biodegradation by laccase and adsorption on immobilized polyimide aerogels surface. As seen, the contribution of CBZ biodegradation by laccase for ultrapure water and secondary effluent was higher than 60% while adsorption resulted in less than 10% of the total removal. It can be concluded that immobilized laccase on polyimide aerogels is a good alternative for CBZ removal with a good removal efficiency after 7 cycles of operation (50% and 65% for spiked water and secondary effluent, respectively). This mechanism could be implemented in the wastewater treatment plant to remove the pharmaceutical compounds present in the environment after the economy and scale-up analysis of the process.

4. **Conclusions**

In this work, surface modified polyimide aerogels were successfully prepared and the immobilization of laccase onto these aerogels was obtained by covalent bonding and adsorption to compare their behavior. FTIR analysis demonstrated the successful modification of polyimide aerogels and enzyme immobilization onto them. Covalent immobilization was more effective than adsorption and was employed for the rest of the study. Results showed that the immobilized laccase on polyimide aerogels possesses significantly improved activity under acidic or basic pH range in comparison with the free enzyme. Furthermore, for all the temperature range the activity of the immobilized enzyme was higher compared to the free enzyme form. The storage stability improved by the immobilization on this support material. The reusability tests towards oxidation of ABTS showed that the immobilized laccase maintained 22% of the initial activity after 7 cycles. Immobilized laccase on polyimide aerogels for CBZ degradation exhibited 76 % and 74% removal in spiked water and secondary effluent, respectively.

Furthermore, after 7 cycles the CBZ removal efficiency remained higher (50% and 65% for spiked water and secondary effluent, respectively).

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